APPENDIX

- **Required Information File**

Detailed and quantitative experimental data of the pharmacological effects of certain compounds that already disclosed in this patent application.

Example 1: Object Recognition Task Model

The cognition-enhancing properties of compounds of this invention were estimated using a model of animal cognition: the object recognition task model.

Male Wister rats (230–280 grams) obtained from N. I. N. (National Institute of Nutrition, Hyderabad, India) were used as experimental animals.. Four animals were housed in each cage. Animals were kept on 20 % food deprivation before one day and given water ad libitum throughout the experiment and maintained on a 12 hours light/dark cycle. Also the rats were habituated to individual arenas for 1 hour in the absence of any objects.

One group of 12 rats received vehicle (1 mL/Kg) orally and another set of animals received compound of the formula (I) either orally or i.p., before one hour of the familiar (T1) and choice trial (T2).

The experiment was carried out in a 50 x 50 x 50 cm open field made up of acrylic. In the familiarization phase, (T1), the rats were placed individually in the open field for 3 minutes, in which two identical objects (plastic bottles, 12.5 cm height x 5.5 cm diameter) covered in yellow masking tape alone (a1 and a2) were positioned in two adjacent corners, 10 cm. from the walls. After 24 hours of the (T1) trial for long-term memory test, the same rats were placed in the same arena as they were placed in T1 trial. Choice phase (T2) rats were allowed to explore the open field for 3 minutes in presence of one familiar object (a3) and one novel object (b) (Amber color glass bottle, 12 cm high and 5 cm in diameter. Familiar objects presented similar textures, colors and sizes. During the T1 and T2 trial, explorations of each object (defined as sniffing, licking, chewing or having moving vibrissae whilst directing the nose towards the object at a distance of less than 1 cm) were recorded separately by stopwatch. Sitting on an object was not regarded as exploratory activity, however, it was rarely observed.

T1 is the total time spent exploring the familiar objects (a1 + a2).

T2 is the total time spent exploring the familiar object and novel object (a3 +b).

The object recognition test was performed as described by Ennaceur, A., Delacour, J., 1988, A new one-trial test for neurobiological studies of memory in rats - Behavioral data, Behav. Brain Res., 31, 47-59.

Some representative compounds have shown positive effects indicating the increased novel object recognition viz; increased exploration time with novel object and higher discrimination index.

Example No.	Discriminative Index
16.	0.76
19.	0.62

20.	0.59
24.	0.55
25.	0.52
30.	0.61
53.	0.64
56.	0.63

Example 2: Water Maze

The water maze apparatus consisted of a circular pool (1.8 m diameter, 0.6 m high) constructed in black Perspex (TSE systems, Germany) filled with water (24 ± 2°C) and positioned underneath a wide-angled video camera to track animal. The 10 cm^2 perspex platform, lying 1 cm below the water surface, was placed in the centre of one of the four imaginary quadrants, which remained constant for all rats. The black Perspex used in the construction of the maze and platform offered no intramaze cues to guide escape behavior. By contrast, the training room offered several strong extramaze visual cues to aid the formation of the spatial map necessary for escape learning. An automated tracking system, [Videomot 2 (5.51), TSE systems, Germany] was employed. This program analyzes video images acquired via a digital camera and an image acquisition board that determined path length, swim speed and the number of entries and duration of swim time spent in each quadrant of the water maze.

Example No.	Scopolamine Induced Reversal
16.	10 mg-kg, p.o.
19.	< 1 mg-kg, p.o.
20.	3 mg-kg, p.o.
24.	3 mg-kg, p.o.
25.	> 10 mg-kg, p.o.
30.	> 10 mg-kg, p.o.
53.	> 10 mg-kg, p.o.
56.	> 10 mg-kg, p.o.

Reference: (A) Yamada N., Hattoria A., Hayashi T., Nishikawa T., Fukuda H. et. Al., Pharmacology, Biochem. And Behaviour, 2004, 78, 787-791. (B) Linder M. D., Hodges D. B., Hogan J. B., Corsa J. A., et al The Journal of Pharmacology and Experimental Therapeutics, 2003, 307 (2), 682-691.

Example 3: T-Maze

Wistar rats weighing 200-220g are acclimatized to the laboratory environment for 7 days. Rats are housed in a group of four in a controlled environment (Temp = 22 ± 2 °C; Humidity = 50 ± 5 %) and maintained on a 12-h light/dark cycle with lights on at 07:00 with 25 % food deprivation and water provided *ad libitum*. T-maze consists of a stem 69 x 10.5 cm, right & left arm, which is 44 x 10.5 cm each, and height of the T-maze is 23 cm. The right & left arm accompanied by sliding guillotine doors of 22 cm in height. Rats are subjected to a forced trial (rat is forced into one of the arm) which is followed by a choice trial (rat has access to both the arm) each trial is carried out for interval of 3 min each days for five days. Test compound is administered after the forced trial, and after a gap of 2 hour rats are subjected to choice trial. Between each trial the T-maze is cleaned with 70 % v/v alcohol. The choice accuracy is calculated individually for each animal.

Example No.	Increase in Choice Accuracy
16.	10 mg-kg, p.o.
19.	< 1 mg-kg, p.o
20.	3 mg-kg, p.o.
24.	3 mg-kg, p.o.
25.	> 10 mg-kg, p.o.
30.	> 10 mg-kg, p.o.
53.	< 10 mg-kg, p.o.

Example 4: Passive avoidance

Animals were trained in a single-trial, step through, light–dark passive avoidance paradigm. The training apparatus consisted of a chamber 300 mm in length, 260 mm wide and 270 mm in height, constructed to established designs. The front and top were transparent, allowing the experimenter to observe the behavior of the animal inside the apparatus. The chamber was divided into two compartments, separated by a central shutter that contained a small opening 50 mm wide and 75 mm high set close to the front of the chamber. The smaller of the compartments measured 9 mm in width and contained a low-power (6V) illumination source. The larger compartment measured 210 mm in width and was not illuminated. The floor of this dark compartment consisted of a grid of 16 horizontal stainless-steel bars that were 5 mm in diameter and spaced 12.5 mm apart. A current generator supplied 0.75 mA to the grid floor, which was scrambled once every 0.5 seconds across the 16 bars. A resistance range of 40–60 micro ohms was calculated for a control group of rats and the apparatus was calibrated accordingly. An electronic circuit detecting the resistance of the animal ensured an accurate current delivery by automatic variation of the voltage with change in resistance.

Experimental procedure:

This was carried out as described previously (Fox et al., 1995). Adult male Wister rats weighing 200-230 grams were used. Animals were brought to the laboratory 1 hour before the experiment. On the day of training, animals were placed facing the rear of the light compartment of the apparatus. The timer was started once the animal has completely turned to face the front of the chamber. Latency to enter the dark chamber was recorded (usually < 20 seconds) and having completely entered the dark compartment an inescapable foot shock of 0.75 mA for 3 seconds was administered to the animal. Animals were then returned to their home cages. Between each training session, both compartments of the chamber were cleaned to remove any confounding olfactory cues. Recall of this inhibitory stimulus was evaluated 24 hours, 72 hours and on 7 day post-training by returning the animal into the light chamber and recording their latency to enter the dark chamber, a criterion time of 300 seconds was employed.

Example No.	Latency to Dark Region Compared to Vehicle (92.72)	
16.	> 10 mg-kg, p.o.	176.6
19.	< 3 mg-kg, p.o.	168.3
20.	3 mg-kg, p.o.	205.9
24.	3 mg-kg, p.o.	232.3
25.	10 mg-kg, p.o.	144.8
30.	< 10 mg-kg, p.o.	207.9
53.	10 mg-kg, p.o.	165.4
56.	> 10 mg-kg, p.o.	105.1
57.	< 10 mg-kg, p.o.	217.3

Reference: (A) Callahan P. M., Ilch C. P., Rowe N. B., Tehim A., Abst. 776.19.2004, Society for neuroscience, 2004. (B) Fox G. B., Connell A. W. U., Murphy K. J., Regan C. M., Journal of Neurochemistry, 1995, 65, 6, 2796-2799.

Example 4: Binding assay for human 5-HT$_6$ receptor

Compounds can be tested according to the following the procedures.

Materials and Methods:

Receptor source: Human recombinant expressed in HEK293 cells

Radioligand : [^{3}H]LSD (60-80 Ci/mmol)

Final ligand concentration - [1.5 nM]

Non-specific determinant : Methiothepin mesylate - [0.1 μM]

Reference compound : Methiothepin mesylate

Positive control: Methiothepin mesylate

Incubation conditions:

Reactions were carried out in 50 µM TRIS-HCl (pH 7.4) containing 10 µM $MgCl_2$, 0.5 mM EDTA for 60 minutes at 37 °C. The reaction was terminated by rapid vacuum filtration onto glass fiber filters. Radioactivity trapped onto the filters was determined and compared to control values in order to ascertain any interactions of test compound(s) with the cloned serotonin 5-HT_6 binding site.

Example No.	Ki (nM)
16.	4.30
19.	1.63
20.	2.16
24.	12.10
25.	7.02
30.	2.49
53.	6.05
56.	2.13
57.	6.00
59.	10.20
116.	98.20
128.	4.48
130.	20.90
132.	46.6

Literature Reference: Monsma F. J. Jr., et al., Molecular Cloning and Expression of Novel Serotonin Receptor with High Affinity for Tricyclic Psychotropic Drugs. Mol. Pharmacol. (43): 320-327 (1993).

Example 5: Rodent Pharmacokinetic Study

Male wistar rats (230 – 280 grams) obtained from N. I. N. (National Institute of Nutrition, Hyderabad, India) were used as an experimental animal.

Three to five animals were housed in each cage. Animals were kept on 20 % food deprivation before one day and given water ad libitum throughout the experiment and maintained on a 12 hours light/dark cycle. One group of rats received NCE compound (3-50 mg/Kg) orally and another group of animals received same compound intravenously.

At each time point blood was collected by jugular vein. Plasma was stored frozen at -20°C until analysis. The concentrations of the NCE compound in plasma were determined using LC-MS/MS method.

Schedule time points: Pre dose 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 hours after dosing (n=3). The NCE compounds were quantified in plasma by validated LC-MS/MS method using solid phase extraction technique. NCE compounds were quantified in the calibration range of 2-2000 ng/ml in plasma and brain homogenate. Study samples were analyzed using calibration samples in the batch and quality control samples spread across the batch.

Pharmacokinetic parameters C_{max}, T_{max}, AUC_t, $T_{1/2}$ & Bioavailability were calculated by non-compartmental model using software WinNonlin version 4.1.

Ex No.	Strain/ Sex	Dose (mg/kg)	Vehicle (in water)	Route of administration	C_{max} (ng/mL)	T_{max} (hr)	AUC_t (ng.hr/mL)	$T_{1/2}$ (hr)	Bioavailability (%)
16.	Wistar / M	10	50 % PEG 400	PO	126 ± 136	1.50 ± 0.71	301 ± 323	1.98 ± 0.72	6
	Wistar / M	10	50 % PEG 400	IV	3547 ± 908	0.12 ± 0.06	5002 ± 620	1.07 ± 0.30	
	Wistar / M	10	50 % PEG 400	IP	569 ± 117	0.71 ± 0.39	1316 ± 433	1.36 ± 0.21	26
	Wistar / M	50	10 % PEG 400	PO	949 ± 238	1.75 ± 0.76	4557 ± 1398	3.97 ± 3.83	49
	Wistar / M	10	10 % PEG 400	IV	1716 ± 140	0.03 ± 0.00	1864 ± 319	0.71 ± 0.00	
19.	Wistar / M	10	50 % PEG 400	PO	88 ± 52	1.80 ± 1.30	402 ± 251	3.52 ± 1.55	26
	Wistar / M	10	50 % PEG 400	IP	150 ± 41	0.74 ± .72	721 ± 176	6.10 ± 3.72	46
	Wistar / M	10	50 % PEG 400	IV	3411 ± 1104	0.03 ± 0.00	1561 ± 71	1.82 ± 0.90	
	Sprague Dawley/	10	50 % PEG 400	PO	61 ± 36	2.00 ±	331 ± 250	11.3 ±	17

	M					1.12		12.65	
	Sprague Dawley/ M	10	50 % PEG 400	IP	190 ± 75	0.72 ± 0.64	793 ± 263	6.32 ± 5.19	41
	prague Dawley/ M	10	50 % PEG 400	IV	4556 ± 1027	0.03 ± 0.00	1917 ± 270	1.82 ± 0.46	
	Wistar / M	50	50 % PEG 400	PO	560 ± 269	3.50 ± 0.55	4406 ± 2131	5.33 ± 1.08	56
	Wistar / M	50	50 % PEG 400	IP	505 ± 107	1.82 ± 0.99	5409 ± 1479	9.43 ± 2.15	68
	Wistar / M	10	50 % PEG 400	IV	3530 ± 1178	0.03 ± 0.00	1579 ± 363	3.42 ± 1.89	
20.	Wistar / M	10	50 % PEG 400	PO	67 ± 17	0.90 ± 0.22	167 ± 35	2.64 ± 1.55	5
	Wistar / M	10	50 % PEG 400	IP	383 ± 90	0.61 ± 0.43	944 ± 444	1.38 ± 0.26	32
	Wistar / M	10	50 % PEG 400	IV	2165 ± 518	0.04 ± 0.02	2978 ± 468	1.33 ± 0.6	
	Sprague Dawley/ M	10	50 % PEG 400	PO	58 ± 17	1.83 ± 1.26	220 ± 82	4.76 ± 4.35	7
	Sprague Dawley/ M	10	50 % PEG 400	IP	1425 ± 366	0.15 ± 0.09	2422 ± 498	1.34 ± 0.25	74
	Sprague Dawley/ M	10	50 % PEG 400	IV	3442 ± 928	0.03 ± 0.00	3272 ± 1044	0.95 ± 0.24	
	Wistar / M	50	10 % PEG 400	PO	355 ± 181	2.50 ± 0.61	1393 ± 630	1.69 ± 0.54	11
	Wistar /	10	10 % PEG	IV	2086 ± 906	0.19	2518 ± 557	1.14	

	M		400			± 0.19		± 0.30	
24.	Wistar/ M	10	50 % PEG 400	PO	121 ± 130	2.08 ± 0.80	452 ± 653	2.17 ± 2.10	24
	Wistar / M	10	50 % PEG 400	IP	1456 ± 679	0.35 ± 0.57	2219 ± 1523	1.39 ± 0.59	118
	Wistar / M	10	50 % PEG 400	IV	2251 ± 630	0.03 ± 0.00	1885 ± 198	1.15 ± 0.40	
	Sprague Dawley/ M	10	50 % PEG 400	PO	47 ± 12	1.50 ± 1.33	145 ± 66	2.83 ± 1.50	5
	Sprague Dawley/ M	10	50 % PEG 400	IP	926 ± 194	0.11 ± 0.07	1462 ± 176	1.35 ± 0.41	55
	Sprague Dawley/ M	10	50 % PEG 400	IV	2527 ± 1291	0.04 ± 0.02	2679 ± 326	1.15 ± 0.24	
	Wistar / M	50	10 % PEG 400	PO	492 ± 296	2.06 ± 0.78	2048 ± 1285	2.48 ± 1.05	22
	Wistar / M	10	10 % PEG 400	IV	1258 ± 332	0.14 ± 0.12	1896 ± 382	1.27 ± 0.50	
25.	Wistar / M	10	10 % PEG 400	PO	267 ± 156	1.38 ± 0.79	1029 ± 594	2.57 ± 1.33	13
	Wistar / M	10	10 % PEG 400	IP	475 ± 153	1.88 ± 1.41	2839 ± 1254	4.44 ± 2.11	37
	Wistar / M	10	10 % PEG 400	IV	2387 ± 995	0.09 ± 0.13	7743 ± 3632	2.66 ± 1.9	
	Wistar /	50	50 % PEG	PO	905 ± 276	3.33	10090 ±	8.19	31

	M		400			± 0.50	2122	± 5.30	
	Wistar / M	10	50 % PEG 400	IV	4055 ± 703	0.03 3 ± 0.00	6509 ± 2206	1.40 ± 0.23	
30.	Wistar / M	10	50 % PEG 400	PO	308 ± 126	1.07 ± 0.61	1190 ± 406	3.12 ± 1.73	8
	Wistar / M	10	50 % PEG 400	IP	577 ± 271	0.91 ± 0.67	3583 ± 1709	4.50 ± 1.29	25
	Wistar / M	10	50 % PEG 400	IV	3551 ± 939	0.05 ± 0.03	14319 ± 13179	2.34 ± 1.21	
	Wistar / M	50	10 % PEG 400	PO	1037 ± 558	3.56 ± 1.66	10255 ± 5028	5.55 ± 1.58	13
53.	Wistar / M	10	10 % PEG 400	PO	1225 ± 379	0.50 ± 0.00	2221 ± 1019	1.90 ± 2.36	21
	Wistar / M	10	10 % PEG 400	IP	1602 ± 518	0.43 ± 0.19	3127 ± 831	0.88 ± 0.20	30
	Wistar / M	10	10 % PEG 400	IV	4970 ± 595	0.14 ± 0.09	10381 ± 1604	0.87 ± 0.10	
	Wistar / M	50	50 % PEG 400	PO	1243 ± 306	1.48 ± 1.24	8676 ± 2919	7.53 ± 5.29	17
56.	Wistar / M	10	50 % PEG 400	PO	982 ± 115	1.20 ± 0.45	3043 ± 651	1.92 ± 1.51	24
	Wistar / M	10	50 % PEG 400	IP	1309 ± 443	0.69 ± 0.36	5142 ± 1591	1.88 ± 1.03	40
	Wistar / M	10	50 % PEG 400	IV	5064 ± 1442	0.05 ± 0.02	12843 ± 6857	1.05 ± 0.14	

	Wistar / M	50	10 % PEG 400	PO	2498 ± 1132	3.07 ± 0.84	30369 ± 16354	9.06 ± 8.03	63
	Wistar / M	10	10 % PEG 400	IV	3158 ± 937	0.36 ± 0.64	9676 ± 3156	1.01 ± 0.30	
57.	Wistar / M	10	50 % PEG 400	PO	1999 ± 835	2.50 ± 0.58	6215 ± 3440	1.09 ± 0.25	47
	Wistar / M	10	50 % PEG 400	IP	2087 ± 553	2.38 ± 2.46	11882 ± 5319	6.32 ± 3.99	89
	Wistar / M	10	50 % PEG 400	IV	3097 ± 357	0.19 ± 0.40	13326 ± 3850	2.28 ± 1.36	
128.	Wistar / M	10	50 % PEG 400	PO	50 ± 42	1.61 ± 2.02	159 ± 73	12.11 ± 11.53	21
	Wistar / M	10	50 % PEG 400	IP	253 ± 67	0.25 ± 0.00	388 ± 160	4.23 ± 1.97	51
	Wistar / M	10	50 % PEG 400	IV	544 ± 150	0.03 ± 0.00	755 ± 376	2.65 ± 0.33	
	Wistar / M	50	50 % PEG 400	PO	227 ± 79	3.00 ± 1.41	1086 ± 297	3.98 ± 2.23	18
	Wistar / M	10	50 % PEG 400	IV	1030 ± 291	0.03 ± 0.00	1224 ± 310	3.85 ± 0.9	

Example 6: Rodent Brain Penetration Study

Male Wister rats (230–280 grams) obtained from N. I. N. (National Institute of Nutrition, Hyderabad, India) was used as an experimental animal.

Three to five animals were housed in each cage. Animals were kept on 20 % food deprivation before one day and given water ad libitum throughout the experiment, and maintained on a 12 hours light/dark cycle. Each group of animals received NCE compound (3-50 mg/Kg) orally or ip.

At each time point blood was collected by jugular vein. Animals will be sacrificed to collect the brain tissue and was homogenized. Plasma and Brain was stored frozen at –20 °C until analysis. The concentrations of the NCE compound in plasma and Brain were determined using LC-MS/MS method.

Schedule time points: Pre dose 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 hours after dosing (n=3). The NCE compounds were quantified in plasma and brain homogenate by validated LC-MS/MS method using solid phase extraction technique. NCE compounds were quantified in the calibration range of 2-2000 ng/ml in plasma and brain homogenate. Study samples were analyzed using calibration samples in the batch and quality control samples spread across the batch.

Pharmacokinetic parameters C_b/C_p & AUC_b/AUC_p were calculated by non-compartmental model using software WinNonlin version 4.1.

Example No.	Strain/ Sex	Dose (mg/kg)	Vehicle (in water)	Route of administration	C_b/C_p	AUC_b / AUC_p
16.	Wistar / M	10	50 % PEG 400	IP	1.70	1.26
19.	Wistar / M	10	50 % PEG 400	PO	0.96	0.34
	Wistar / M	10	50 % PEG 400	IP	3.89	2.41
	Sprague Dawley/ M	10	50 % PEG 400	IP	1.43	1.48
20.	Wistar / M	10	50 % PEG 400	IP	1.94	0.56
24.	Wistar / M	10	50 % PEG 400	IP	2.44	3.57
	Sprague Dawley/ M	10	50 % PEG 400	IP	2.63	2.57
25.	Wistar / M	10	10 % PEG 400	IP	2.83	2.71
30.	Wistar / M	10	50 % PEG 400	IP	1.71	2.12
53.	Wistar / M	10	10 % PEG 400	IP	0.84	0.77
56.	Wistar / M	10	50 % PEG 400	IP	0.87	0.66
57.	Wistar / M	10	50 % PEG 400	IP	2.17	1.03
128.	Wistar / M	10	50 % PEG 400	IP	0.83	5.31